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(19) (CA) **CANADIAN PATENT** (12)

(54) Human Anti-Rh(D) Monoclonal Antibodies

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ABSTRACT

Human Anti-Rh(D) Monoclonal Antibodies

The present invention provides human monoclonal antibodies having the following essential

5 characteristics:

- (a) exhibiting activity against Rh(D) antigen, but not C, c, E or e antigens of the Rh blood group system;
- (b) being IgG1 proteins;
- (c) having kappa light chains;
- 10 (d) being G1m (3) or G1m (1, 17) allotype;
- (e) exhibiting activity against D^u cells by an indirect antiglobulin test;
- (f) exhibiting activity against D^{IV}, D^V and D^{VII} variant antigens; and
- 15 (g) being inactive against D^{VI} or D^B variant antigens, which may be employed for Rh-typing of red blood cells and passive immunisation to prevent haemolytic disease of the newborn.

20 Cell lines capable of producing such monoclonal antibodies were deposited at the European Collection of Animal Cell Cultures, Porton Down, U.K. on 16th September 1987 under accession numbers ECACC 87091605 and ECACC 87091604.

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CLAIMS:

1. A human monoclonal antibody having the following essential characteristics:
- 5 (a) exhibiting activity against Rh(D) antigen, but not C, c, E or e antigens of the Rh blood group system;
- (b) being IgG1 proteins;
- (c) having kappa light chains;
- (d) being G1m (3) or G1m (1, 17) allotype;
- 10 (e) exhibiting activity against D^u cells by an indirect antiglobulin test;
- (f) exhibiting activity against D^{IV}, D^V and D^{VII} variant antigens; and
- (g) being inactive against D^{VI} or D^B variant antigens
- 15 and antigen-binding fragments thereof.
2. A monoclonal antibody as claimed in claim 1 selected from the monoclonal antibodies of the cell lines ECACC 87091605 and ECACC 87091604.
- 20 3. An anti-Rh(D) reagent wherein a monoclonal antibody as claimed in claim 1 is combined with one or more further anti-Rh(D) antibodies having one or more additional binding specificities.
- 25 4. An anti-Rh(D) reagent as claimed in claim 3 wherein a monoclonal antibody capable of binding the D^{VI} variant is present.
- 30 5. An anti-Rh(D) reagent as claimed in claim 3 wherein the antibody component comprises:
- (a) a monoclonal antibody as defined in claim 1, and
- (b) an IgM anti-Rh(D) with no or only weak anti-D^u activity. /
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6. An anti-Rh(D) reagent as claimed in claim 5,
wherein the antibody component additionally comprises an
IgG monoclonal anti-Rh(D) capable of exhibiting activity
against D^{VI} cells in an indirect antiglobulin test.
- 5
7. An anti-Rh(D) reagent as claimed in claim 5 wherein
component (b) is selected from the monoclonal IgMs of
the deposited hybridoma cell lines MAD-2 (ECACC
86041803) and FOM-1 (ECACC 87021301).
- 10
8. A human lymphocyte-derived cell line capable of
producing a monoclonal antibody as claimed in claim 1.
9. A cell line as claimed in claim 8 selected from
- 15 ECACC 87091605 and ECACC 87091604.
10. A monoclonal antibody as claimed in claim 1 for use
in passive immunisation to
prevent haemolytic disease of the newborn.
- 20
11. A monoclonal antibody as claimed in claim 10 of the
allotype G1m(3).
12. A culture supernatant obtained by cultivation of a
- 25 cell line as claimed in claim 8 or claim 9.
13. A pharmaceutical composition for use in passive
immunisation to prevent haemolytic disease of the
newborn comprising a monoclonal antibody as claimed in
- 30 claim 1 in association with a physiologically acceptable
carrier or diluent.
14. A pharmaceutical composition as claimed in claim 13
wherein said monoclonal antibody is of the allotype
- 35 G1m(3).



15. A pharmaceutical composition as claimed in claim 13 wherein the antibody component additionally comprises a monoclonal antibody capable of binding the D^{VI} variant.
- 5 16. A pharmaceutical composition as claimed in claim 13 wherein the antibody component includes one or more anti-Rh(D) antibodies of the IgG3 sub-class.
- 10 17. A method of Rh-typing wherein a monoclonal antibody as claimed in claim 1 or an anti-Rh(D) reagent as claimed in claim 3 is employed, said monoclonal antibody or said reagent being in the form of an aqueous solution.

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PATENT AGENTS

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PK 51-900 (Kumpel 1)

Human Anti-Rh(D) Monoclonal Antibodies

The present invention relates to human monoclonal antibodies to the Rh(D) antigen of human red blood cells. In particular, it relates to such antibodies of the IgG1 sub-class which may be used to detect not only the normal Rh(D) antigen on either D-positive or "weak D" or D^u cells, but also important variants of the Rh(D) antigen.

Of the antigens of the so-called Rh blood group system, the Rh(D) antigen is responsible for some of the most severe reactions following transfusion to a patient with corresponding antibody. Since an Rh(D-) individual with anti-Rh(D) who receives Rh(D+) blood is liable to suffer substantial red blood cell (RBC) destruction due to the Rh(D) phenotype incompatibility, blood of donors and blood transfusion recipients is routinely classified as Rh(D+) or Rh(D-) by agglutination tests with anti-Rh(D) antibody. The Rh phenotype of RBCs is commonly further defined with reference to the Fisher-Race system, which is based on the assumption that the inheritance of the Rh antigens is determined by three pairs of allelic genes, C-c, D-d and E-e, acting at very closely linked loci. According to this theory, a person may inherit a set of three Rh genes from each of his parents (i) C or c, (ii) D or d, (iii) E or e (no d antigen has as yet been identified, but the symbol 'd' is used to indicate the presence of a gene, allelic to the D gene, which does not produce D antigen). For example, an Rh(D+) person may inherit CDe from one parent and cde from the other. The frequencies of the commonest Rh gene combinations as determined with



reference to the Fisher-Race system for an English population, together with the 'short symbols' which are used, particularly in speech, are given in Table 1 below.

5

Table I
Frequency of common Rh genes for an English Population

	Short symbol	CDE nomenclature	Frequency (%)
10	\underline{R}^1	<u>CDe</u>	40.8
	\underline{r}	<u>cde</u>	38.9
	\underline{R}^2	<u>cDE</u>	14.1
	\underline{R}^0	<u>cDe</u>	2.6
15	\underline{R}^{1w}	<u>C^wDe</u>	1.3
	\underline{r}''	<u>cdE</u>	1.2
	\underline{r}'	<u>Cde</u>	0.01
	\underline{R}^Z	<u>CDE</u>	} rarer
	\underline{r}^Y	<u>CdE</u>	
20	\underline{R}^N	<u>(C)D(e)</u>	

Despite expansion over the years, the Fisher-Race system has not been adequate to account for all the reactions that have been observed with the Rh system (Mollison, P.L. (1983) Blood Transfusion In Clinical Medicine, 7th edn., Blackwell Scientific, Oxford). Nevertheless, the World Health Organisation has recommended that in the interest of simplicity and uniformity this nomenclature should be universally adopted and all Rh genotypes given hereinafter are defined on the basis of the conventional Fisher-Race system.

In addition to the need for anti-Rh(D) antibody for Rh-typing of RBCs, such antibody is also importantly required for passive immunisation of Rh(D-) mothers to prevent haemolytic disease of the newborn (HDN). This condition arises in newborn Rh(D+) infants of Rh(D-) mothers previously sensitized to Rh(D)

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antigen as a result of IgG anti-Rh(D) antibodies crossing the placenta during pregnancy and causing foetal RBC destruction. Sensitization of the Rh(D-) mother to Rh(D) antigen may have occurred at the birth of an earlier Rh(D+) child due to some foetal RBCs entering the maternal circulation and being recognised by the maternal immune system. To reduce the incidence of HDN, it is routine practice in the United Kingdom and many other countries to give anti-Rh(D) antibodies to Rh(D-) mothers immediately after the birth of an Rh(D+) infant so that any Rh(D+) RBCs which have entered the maternal circulation are rapidly removed (Mollison, P.L. (1983) loc. cit.; Laros Jr., R.K. (1986), "Erythroblastosis Fetalis" in "Blood Group Disorders In Pregnancy", Ch. 7, p. 103).

At the present time, anti-Rh(D) antibody for use in both Rh-typing of RBCs and passive immunisation of Rh(D-) mothers is largely obtained directly from female donors immunised during pregnancy or from immunised male volunteers. The success of the programme of post-partum prophylactic administration of human anti-Rh(D) immunoglobulin to Rh(D-) women has, however, resulted in a dramatic reduction in the number of naturally alloimmunised women (Urbaniak, S.J., "RhD haemolytic disease of the newborn: the changing scene", Br. Med. J. (1985) 291, 4-6). Also, deliberate immunisation of individuals with Rh(D+) RBCs carries the risks common to receiving any transfusion of RBCs, e.g. risk of transmission of hepatitis viruses and HIV. Hence, there is much interest in obtaining human monoclonal anti-Rh(D) antibodies for both diagnostic and therapeutic purposes.

As stated above, in routine blood testing, blood types are divided into Rh(D+) and Rh(D-) on the basis of the apparent presence or absence

of Rh(D) antigen on the RBCs as indicated by agglutination tests with anti-Rh(D). However, a small number of persons with apparently Rh(D-) blood have RBCs that are not directly agglutinated by anti-Rh(D) during such routine testing, but that do react when the D-typing test is performed using selected anti-Rh(D) reagents by the indirect antiglobulin test. Cells thus identified are designated D^u. The frequency of the D^u phenotype is about 0.2% overall, 0.6% among Caucasians, and about 1.5% of all Rh(D-) gravid women. At least three different mechanisms may be responsible for the expression of the D^u phenotype: (1) hereditary absence of a portion of the complete Rh(D) antigen, (2) gene interaction with suppression of D by C in the trans position, and (3) a D gene producing a weak antigen.

In the early 1950s, reports first appeared of the presence of anti-Rh(D) in individuals of the D^u phenotype following blood transfusion with Rh(D+) blood or pregnancy resulting in the birth of a Rh(D+) infant. It later became apparent that in some individuals whose blood is classified Rh(D+) parts of the Rh(D) antigen are missing from the RBCs. When exposed by transfusion or pregnancy to Rh(D+) RBCs carrying the complete Rh(D) antigen, persons carrying an incomplete Rh(D) antigen on their RBCs are capable of making alloanti-D against the Rh(D) antigen portion they lack. The blood of such individuals is called D variant when the RBCs react directly with routine anti-Rh(D) reagents or D^u variant when the cells react only by the indirect antiglobulin technique.

The observation that allo anti-Rh(D) can be produced in patients who have Rh(D+) RBCs has led to common usage of the term "D mosaic" to describe the Rh(D) antigen in its complete native form. Routine anti-Rh(D) reagents generally cannot differentiate

those RBCs that lack part of the D mosaic from those that have all the D components.

The D variant phenotypes have been categorised by Tippett and Sanger (Vox. Sang. (1962) 2, 9-13). This system is based on the interaction of RBCs and serum from D- and D^u variant individuals. The seven categories (see Table II below) allow for expansion; subdivisions are already recognised in categories III< IV and V. Categories I and II have been found to have so many similarities that they are now generally considered as a single sub-group.

Table II

Tippett and Sanger categories for D- or D^u positive blood with anti-Rh(D)

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Category	Racial origin	Usual haplotype
I	White	<u>D_{Ce}</u>
20 II		
IIIa	Black	
IIIb	Usually Black	<u>D_{Ce}</u>
IIIc	White	
IVa	Mostly Black, some White	
25 IVb	White	<u>D_{Ce}</u>
Va		Black and White
Vb	White	<u>D^u_{Ce}</u>
Vc	Black and White	
VI	Nearly all White	<u>D^u_{Ce}</u>
30 VII	White	<u>D_{Ce}</u>

An alternative, but lesser used, classification by Wiener uses letters A,B,C,D instead of Roman Numerals. Although there is no direct correlation between the two systems, it is often considered that D^B and D^{Vl} are interchangeable.

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Although the frequency of D and D^u variant individuals within the human population is relatively

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low, the total number of individuals of these blood types who potentially have some risk of effective anti-Rh(D) formation as a result of exposure by blood transfusion or pregnancy to non-variant Rh(D+) cells is far from insignificant. Moreover, in addition to Rh(D-) women who give birth to Rh(D+) or D^u infants, D^u variant women who give birth to an Rh(D+) infant may also benefit from post-partum anti-Rh(D) treatment to reduce the risk of HDN (White, C.A. et al. (1983) Am. J. Obstet. Gynecol. 145, 1069-1073). Anti-sera capable of distinguishing D and D^u variant RBCs are not widely available. Hence, provision of anti-Rh(D) monoclonal antibodies with a range of binding specificities for D and D^u variant RBCs is seen as useful in enabling the more ready identification and categorisation of individuals possessing such cells (especially D or D^u variant pregnant females who are suitable candidates for prophylactic anti-Rh(D) treatment) as well as for providing further structural information on the Rh(D) antigen complex.

Human monoclonal anti-Rh(D) antibody production has previously been achieved by:-

- (a) directly cloning Epstein Barr virus transformed B lymphocyte cell lines (hereinafter referred to as EBV-transformed LCL) derived from B lymphocytes of anti-Rh(D) positive donors (see GB-A 2127434 of Crawford, published 11 April 1984; Crawford et al. (1983) Lancet 1, 386-388 and Paire et al (1986) Immunol. Lett. 13, 137-141),
- (b) cloning hybridoma cell lines formed by fusing anti-Rh(D) producing, EBV-transformed LCL with mouse, mouse-human or human myeloma cell lines (see co-pending British application of Central Blood Laboratories Authority, published under no. GB-A-2189506 on 28 October 1987, Thompson et al. (1986) Immunol. 58, 157-160 and International Patent Application publication No. WO85/02413 of The Board of Trustees of the Leland

Stanford Jr. University, published on 6 June 1985), or
(c) by fusion of a human LCL with immune B cells (Lowe
et al (1986) Vox. Sang. 51, 212-216).

By cloning EBV-transformed LCL from 3 anti-Rh(D)
5 positive donors, we have been able to obtain, however,
monoclonal anti-Rh(D) antibodies of the IgG class which
have a particularly useful binding specificity spectrum
not shown for any previously disclosed anti-Rh(D)
monoclonal antibody reagent.

10 According to the present invention, we provide
human monoclonal antibodies having the following
essential characteristics:

- (a) exhibiting activity against Rh(D) antigen, but not
C, c, E or e antigens of the Rh blood group system;
- 15 (b) being IgG1 proteins;
- (c) having kappa light chains;
- (d) being G1m (3) or G1m (1, 17) allotype;
- (e) exhibiting activity against D^u cells by an indirect
antiglobulin test;
- 20 (f) exhibiting activity against D^{IV}, D^V and D^{VII}
variant antigens; and
- (g) being inactive against D^{VI} or D^B variant antigens,
and antigen-binding fragments thereof.

Such monoclonal antibodies can be employed as
25 routine anti-Rh(D) reagents to classify RBCs as Rh(D+),
D^u or Rh(D-).

For this purpose, a monoclonal antibody of the
present invention may be employed either alone or in
combination with one or more further anti- Rh(D)
30 antibodies, preferably monoclonal antibodies, having one
or more additional binding specificities. Thus, for
example, a monoclonal antibody of the present invention
may be advantageously blended with a further monoclonal
antibody capable of binding the D^{VI} variant to provide
35 an anti-Rh(D) reagent of broader specificity capable of
classifying D^{VI} variant RBCs as D-positive. In such an
anti-Rh(D) reagent, an IgG1 antibody of the present

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invention may, for example, be combined with an IgG1 monoclonal antibody of the type disclosed in our International patent application published under No. W089/02443 on 23 March 1989 having the following binding characteristics:

- 5 (a) exhibiting activity against Rh(D) antigen, but not C, c, E or e antigens of the Rh blood group system;
- 10 (b) exhibiting activity against D^V , D^{VII} D^{VI} and D^B variant antigens; and
- (c) being substantially non-reactive with non-papain treated D^{IV} cells in an IAG test.

Amongst such antibodies, particularly preferred for use in combination with an anti-Rh(D) monoclonal antibody of the present invention is the monoclonal antibody designated B7 deposited at the European Collection of Animal Cell Cultures, Porton Down, U.K. under accession No. ECACC 87091603 on 16th September 1987.

20 If monoclonal antibodies of the present invention are used for Rh-typing in parallel with an anti-Rh(D) reactive against the D^{VI} variant, eg. an appropriate polyclonal anti-Rh(D) serum, those blood samples giving a positive result in an agglutination test with the latter, but negative results with a monoclonal antibody 25 of the invention can be predicted to be mainly or entirely of the D^{VI} category (since this is virtually the only D variant antigen against which the new monoclonal antibodies are inactive). It has been 30 established that amongst individuals classified as Rh(D+) or D^u by a conventional agglutination test, but who are capable of making anti-Rh(D), a high percentage have the D^{VI} variant antigen (Mollison, P.L. (1983) in "Blood Transfusion In Clinical Medicine", Ch. 8, p 339). 35 One use of the monoclonal antibodies of the invention is, indeed, in investigating the incidence of individuals of the D^{VI} type in the population.

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A monoclonal antibody of the present invention may also be of particular value for use in an anti- Rh(D) typing reagent to supplement the specificity of an anti-Rh(D) with no or only weak anti-D^u activity, i.e. insufficient activity against D^u cells to be able to reliably distinguish such cells from D-negative cells in a conventional agglutination test. Indeed, under FDA regulations in the U.S.A. governing commercial anti-Rh(D) typing reagents, it is obligatory for such a reagent to be able to distinguish D^u RBCs from truly D-negative RBCs. Especially preferred amongst combination anti-Rh(D) reagents of the present invention are such reagents satisfying the above condition wherein an IgG anti- Rh(D) of the invention is employed together with an IgM anti-Rh(D) with no or only weak D^u activity, e.g. an IgM monoclonal anti-Rh(D) selected from the monoclonal IgMs of the deposited hybridoma cell lines MAD-2 (ECACC 86041803) and FOM-1 (ECACC 87021301), which form inter alia, the subject matter of published European Patent Application 0251440 of Central Blood Laboratories Authority, published on 7 January 1988. Such a reagent will advantageously be further complemented with at least one further IgG monoclonal anti-Rh(D) antibody which individually exhibits activity with D^u red cells by the indirect antiglobulin test, such that the blended reagent reacts by the same test with D^u, D^{IV}, D^V and D^{VI} cells. When Rh typing is carried out with such a reagent, D-positive cells will firstly be directly agglutinated by the IgM anti-Rh(D). The remaining non-agglutinated cells (apparently D-negative) may then be subsequently divided into truly D-negative and D^u cells by addition of conventional Coomb's reagent for an indirect antiglobulin test, whereupon D^u cells binding IgG antibody will be agglutinated and thus distinguished.

The monoclonal anti-Rh(D) antibodies of the invention can be made by conventional methods known for

the production of monoclonal antibodies and in particular by the culture of EBV-transformed human B-lymphocytes selected on the basis of secretion of anti-Rh(D) immunoglobulin having the characteristics set
5 out above for the required antibodies. The culture supernatants so produced constitute a further feature of the present invention.

We have now investigated in detail 9 cloned EBV-transformed LCL which produce IgG1 anti-Rh(D)
10 monoclonal antibodies as defined above. All these cloned cell lines were obtained by starting with peripheral B lymphocytes from 2 chosen anti-Rh(D) donors and employing the procedure described in GB-A 2145113 of UK Secretary of State for Social Services, published on
15 20 March 1985 or a substantially similar procedure to establish and clone EBV-transformed LCL producing a monoclonal antibody of the desired specificity (see Example 1). In continuous culture using RPMI- 1640 medium supplemented with 10% (v/v) mycoplasma free-fetal
20 calf serum, 0.2 mg/ml arginine and antibiotics to prevent mycoplasma growth, they have been found to be highly stable and to provide a culture supernatant having an anti-Rh(D) titre, as determined by an indirect antiglobulin (IAG) assay in low ionic strength saline
25 versus R_1R_1 (CDe/CDe) RBCs, in the range 2000-8000. Such a culture supernatant is suitable for use in Rh-typing without the need for concentration and indeed may be diluted for use. Four of the selected cell lines (A1, A2 and A3 derived from a donor A) have been shown

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to maintain a stable anti-Rh(D) titre in continuous culture for over 2 years. Four further cell lines capable of providing a culture supernatant as above (B1, B2, B3 and B11 derived from a donor B) have been maintained in continuous culture for over 8 months without substantial decrease of anti-Rh(D) titre. Antibody production characteristics of the above-mentioned specific clones in continuous culture are summarised in Tables IIIa and IIIb below.

Table IIIa

	IAG titre (3% RBCs in low ionic strength saline)	
15	R ₁ r cells	64-512
	R ₁ R ₁ cells	2000-8000
	R ₁ ^u r cells	8-512
20	Microtitre with bromelain-treated R ₁ R ₂ cells	33,000-131,000
25	Anti-Rh(D) (IU/ml)	10-98
	IgG (µg/ml)	3.9-11.0
30		

Table IIIfb

culture	A1	A2	A3	B1	B2	B3	B11	B12	B13
Supernatant	IAG reactivity of supernatant with 3% RBCs in low ionic strength saline. (Grade: 0 to 6)								
RBC phenotype									
R_1R_1	6	6	6	6	6	6	6	6	6
R_1u_r	6	6	6	6	6	4	5	6	6
$R_{O_r}^u$	6	6	6	6	6	6	6	6	6

It was further found that those monoclonal antibodies of the invention that were tested reacted by IAG with RBCs of phenotypes R_2rG- , hr^s- , R_1R_2 and R_2R_2 but are negative with r^uGr , r^m , r^G , r^s_r , hr^B- , r^w_r and Rh33+.

- 5 Gm allotyping of the antibodies of clones A1, A2, A3, B1, B2, B3, B11, B12 and B13 has shown that they fall into two allotype groups. Using the W.H.O. (1974) notation, the IgG1 anti-Rh(D) antibodies of clones A1, A2 and A3 were found to be of the Glm (1, 17) allotype, whereas the antibodies of the remaining above-mentioned clones derived from donor B were found to be of the allotype Glm(3). The latter antibodies are of particular interest from the point of view of providing a monoclonal
- 10 antibody preparation for use in post-partum immunisation of Rh(D-) mothers. In general, the antibodies in the Glm (1, 17) allotype group were active against the \bar{R}^N antigen, whereas those of Glm(3) allotype were not.

- 20 IgG1 anti-RhD antibodies are generally poor promoters of phagocytosis of Rh(D+) RBCs by monocytes

and macrophages. However, we have found that IgG1 anti-Rh(D) antibodies of the G1m(3) allotype, e.g. the IgG1 antibodies of clones B1, B2, B3, B11, B12 and B13, unlike IgG1 anti-Rh(D) antibodies of the G1m(1,17) allotype, are highly effective in mediating lysis of sensitised RBCs by K lymphocytes in an antibody-directed cell cytotoxicity (ADCC) assay. This is in keeping with the previously reported observation of Parinaud et al. that foetal haemolysis is more severe with maternal IgG1 antibodies of the G1m(4) allotype, i.e. G1m(3) allotype according to the W.H.O (1974) nomenclature (Am. J. Obstet. Gynecol (1985) 1111-1115).

Thus, according to a further aspect of the present invention, we provide a monoclonal IgG1 antibody of the present invention, preferably a monoclonal antibody of the present invention having the allotype G1m(3), for use in passive immunisation of an Rh(D-) or D- or D^u-variant mother after the birth of an Rh(D+) child to prevent sensitisation of the mother to Rh(D) antigen. A sterile solution of such an antibody for human injection may be formulated in any physiologically acceptable aqueous medium, for example isotonic phosphate buffered saline or serum. Alternatively, the antibody may be supplied in a freeze-dried formulation ready for reconstitution prior to use. To provide a highly efficient prophylactic preparation for use in the prevention of HDN, a monoclonal anti-Rh(D) of the present invention, especially such an antibody of the G1m(3) allotype, may desirably be employed with one or more further anti-Rh(D) antibodies, for example one or more further anti-Rh(D) antibodies promoting phagocytosis of Rh(D+) RBCs in vivo, e.g. an anti-Rh(D) monoclonal antibody of the IgG3 sub-class, such as an IgG3 monoclonal anti- Rh(D) of our co-pending Canadian Application No. 577600 of



even date herewith. The aforementioned IgG3 monoclonal anti-Rh(D) antibodies are exemplified by the monoclonal antibody of the deposited cell line ECACC 87091606. For routine use, ideally a prophylactic pharmaceutical composition of the present invention will include an anti-D^{VI} antibody.

According to a still further aspect of the present invention, we provide a method of Rh-typing of RBCs wherein an aqueous solution of a monoclonal anti-Rh(D) immunoglobulin of the present invention is employed. The monoclonal immunoglobulin is preferably contained in a culture supernatant which may be used directly or, more usually, after dilution. Particularly preferred for use in Rh-typing are culture supernatants containing monoclonal anti- Rh(D) immunoglobulin according to the present invention which will agglutinate at high dilution (e.g. 1:1000 dilution) enzyme-treated RBCs carrying the Rh(D) antigen and in the IAG test will agglutinate D^u RBCs at, for example, 1:10 dilution.

As hereinbefore indicated, it may be desirable to blend an IgG1 antibody of the present invention with one or more further anti-Rh(D) monoclonal antibodies of different specificity, e.g. a further IgG1 antibody having anti-D^{VI} activity. Suitable diluents include physiological saline or phosphate buffered saline, advantageously containing bovine serum albumin and a surfactant or suspending agent such as Tween 80 (Trade Mark) or methyl cellulose.

The cell lines A3 and B2 were deposited on 16th September 1987 at the European Collection of Animal Cell Cultures, Porton Down, U.K. under accession numbers ECACC 87091605 and ECACC 87091604 respectively.

Further details of the preparation of the above-noted deposited cell lines of the present

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invention and the identifying characteristics of the culture supernatants obtainable by continuous culture of these are provided in Example 1 of the following non-limiting examples.

EXAMPLE 1(i) Establishment and Cloning of Anti-Rh(D) Producing
EBV-transformed LCL

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(a) Sources of B Lymphocytes

Donor A: female, immunised during her first
and only pregnancy (which resulted in delivery
10 of a normal Rh(D+) infant), boosted with 0.5 ml
packed Rh(D+) (R_2r) RBCs 4 years after parturition,
and a peripheral blood sample obtained 8 days after
boosting when her serum anti-D level was 60IU/ml.

Donor B: male, initially immunised by transfusion
15 in 1966, boosted 6 times since and last boosted
13 days before donating a "buffy coat" fraction
(white cells) in 1985 when his serum anti-D level
was 318IU/ml.

20 (b) Establishment of cell lines

Peripheral blood mononuclear cells from donor
B were separated on Lymphoprep (Nyegaard and Co),
incubated in the presence of EBV (1 ml culture
25 supernatant from filtered mycoplasma free B95-8
cell line per 10^7 cells) at 37°C for 1 hour and
washed in phosphate-buffered saline (PBS). Aliquots
were either

(a) plated at 0.5×10^6 cells/ml in 2 ml wells
30 using lymphoblastoid cell culture medium (RPMI-
1640 medium containing 10% (v/v) mycoplasma free
foetal calf serum (FCS), 0.2 mg/ml arginine, 100 IU/ml
penicillin (Glaxo), 50 µg/ml streptomycin (Glaxo),
25 IU/ml polymixin (Glaxo), 25 µg/ml kanamycin
35 (Gibco), 20 µl/ml fungizone (Squibb), 25 µg/ml
gentamycin sulphate (Sigma) and 20 µg/ml trobicin
(UpJohn)), supplemented with either 1% (v/v) phytohaemag-

glutinin (PHA) or 0.5 µg/ml cyclosporin A (CsA);
or

(b) enriched for surface anti-D positive lymphocytes
by rosetting with bromelain-treated OR₁R₂ (CDe/cDE)

5 RBCs prior to plating out as above.

Four cell lines from donor B were set up:
LCL and enriched LCL with either initial PHA or
CsA supplementation.

Isolated peripheral blood mononuclear cells
10 from donor A were similarly infected with EBV.
After washing with PBS, the cells were resuspended
in lymphoblastoid cell culture medium supplemented
with 1% (v/v) PHA and dispensed into 2 ml wells
above a feeder layer of mouse peritoneal macrophages.

15 All the cultures were subsequently incubated
at 37°C in 5% CO₂, 95% humidified air. Medium
changes were performed every 3 to 4 days and, after
3 weeks culture, the cells were transferred to
50 ml flasks. All the cell lines (except that
20 giving rise to clone B3) were enriched by rosetting
at 3-4 weekly intervals.

(c) Cloning

25 Cells were plated out at limiting dilution
at 5 and 10 cells per well in flat bottomed 96-
well plates over a feeder layer of mouse peritoneal
macrophages (Doyle et al. (1985) Human Immunology
13, 199-209). Cultures were fed once a week and
30 after 3-4 weeks cloned cells positive for anti-
D were grown up.

(d) Derivation of clones

35

Three clones producing anti-Rh(D) antibody
of the IgG1 sub-class (designated A1, A2 and A3)

were derived from B lymphocytes of donor A. The polyclonal cell line giving rise to these clones was initiated with PHA and sequentially enriched for anti-Rh(D) positive cell by rosetting 13 times before cloning.

Five further clones producing anti-Rh(D) antibody of the IgG1 sub-class (designated B₁, B₂, B₃, B11 and B12) were derived from B lymphocytes of donor B via a polyclonal cell line initiated with PHA. B1 and B2 were derived from cell lines repeatedly (6x) selected by rosetting after establishing an LCL. B11 and B12 were derived from a cell line rosetted only twice and B3 was produced from an LCL maintained for 1 year without rosetting before cloning. [It should be noted that B11 was erroneously designated C1 in the priority application for this case (No. GB-A 8722018) of Central Blood Laboratories Authority, filed 18 September 1987].

A further anti-Rh(D) IgG1 producing clone (B13) was derived via a donor B polyclonal cell line supplemented with CsA. This cell line was established from lymphocytes selected for anti- Rh(D) immediately after transformation by EBV and was subsequently re-rosetted four times.

The results of tissue type and karotype analysis of the above selected cell lines are set out in Table IV below.

1A

Table IV

Cell Line	A1	A2	A3	B1	B2	B3	B11	B12	B13
5	A			B					
	Female			Male					
	<u>Tissue Type</u>								
	HLA: A			1,3					
	B			8,35					
10	DR			1,3					
	<u>Karotype</u>								
	sex chromosomes			xy					
	ploidy			most cells					
	diploid			diploid					
15									

(e) Quantitation of anti-Rh(D) activity and IgG in culture supernatants

Anti-Rh(D) activity in the supernatants was quantified against British national standards by Auto Analyser. The mean of at least two determinations was calculated. The quantitative estimation of IgG was performed by ELISA (modification of the method of Wakefield et al. in Clin. Chim. Acta. (1982) 123, 303-310) with at least eight determinations for each supernatant. Coating antibody (affinity purified goat anti-human IgG (Sigma)) was used at 1/200 in 0.05M carbonate buffer pH 9.6. Supernatants and standard (purified human IgG (Sigma)) were diluted in RPMI 1640 + 10% FCS. Peroxidase-conjugated goat anti-human IgG (Sigma) was diluted 1/500 in PBS + 0.05% Tween 20 (Trade Mark) and the substrate was TMB (3,3', 5,5'-tetramethyl benzidine).

Not all the cell lines established under different experimental conditions showed stable antibody production. However, all the cell lines

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which were subsequently cloned had maintained high titres (over 1/33,000 by microtitre) of anti-D for over 6 months. All clones from these cell lines were positive for anti-D and maintained their titres

- 5 throughout the duration of continuous culture (A1, A2 and A3 - over 2 years; B1, B2, B3 and B11 - over 8 months). The doubling time was 3-7 days. The cells grew well in suspension culture with no loss of antibody production.

10

(f) Immunoglobulin class and subclass determination

- 15 An immunodot assay (McDougal et al. J. Imm. Meth, (1983) 63, 281-290) was used to determine the reaction of the monoclonal anti-Rh(D) antibodies absorbed to nitrocellulose with anti-IgG, anti-IgM, anti-kappa and anti-lambda antiserum (Serotec); positive reactions were detected with peroxidase-conjugated anti-sheep IgG
- 20 (Serotec) followed by colour development with 4-chloro-1-naphthol. The IgG subclass was evaluated by agglutination of anti-D coated RBCs by monoclonal anti-subclass antibodies (Unipath).

- 25 The presence of single discrete bands for antibody derived from the selected cell lines after SDS-polyacrylamide gel electrophoresis was evidence of monoclonality.

(g) SDS-Page and Western Blotting.

- 30 Iscove's supernatants (serum free) were electrophoresed under reducing conditions on 15% polyacrylamide gels (Laemmli, Nature (1970) 227, 680-685). The separated proteins were then electrophoretically transferred to nitrocellulose
- 35 membranes (Burnette, Annals Biochem. (1981) 112, 195-203), which were probed with anti-IgG antiserum (Serotec) and detected as above.

AI

(h) Protein A absorption

2 ml volumes of supernatants were run
twice down a 25 mm (1ml) column of Protein A Sepharose
Cl-4B (Trade Mark) (Sigma) and absorption of anti-Rh(D)
5 assessed by titration.

(i) Gm allotyping

RBCs were coated with the monoclonal anti- D
10 antibodies and agglutination assessed using panels of Gm
allotyping reagents (Birmingham or Amsterdam).

The Gm allotypes of the monoclonal antibodies of
clones A1, A2, A3, B1, B2, B3, B11, B12 and B13 together
with other characteristics are given in Table V below.

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Table V

Identification of immunoglobulin class,
subclass, and Gm allotype

Monoclonal antibody:	A1	A2	A3	B1	B2	B3	B11	B12	B13
Anti-IgG:	+++	+++	+++	+++	+++	+++	+++	+++	+++
anti-IgM:	-	-	-	-	-	-	-	-	-
anti-kappa:	+++	+++	+++	+++	+++	+++	+++	+++	+++
anti-lambda:	-	-	-	-	-	-	-	-	-
Anti-IgG1:	6	6	6	6	6	6	6	6	6
anti-IgG2:	0	0	0	0	0	0	0	0	0
anti-IgG3:	0	0	0	0	0	0	0	0	0
anti-IgG4:	0	0	0	0	0	0	0	0	0
Heavy chain									
M _r (kDa):	55.5	55	55	54	54	54	55	*	*
Protein A									
absorption	+++	+++	+++	+++	+++	+++	+++	+++	+++
Gm allotype:	G1	G1	G1	G1	G1	G1	G1	G1	G1
	m(1,17)	m(1,17)	m(1,17)	m(3)	m(3)	m(3)	m(3)	m(3)	m(3)
	Km-1	Km-1	Km-1	Km-1	Km-1	Km-1	Km-1	Km-1	Km-1

* not determined

(ii) Serology

Anti-D titration was performed by adding 50 μ l of 0.1% suspension of bromelain-treated OR_1R_2 (CDe/cDe) erythrocytes to 50 μ l of supernatant, neat and serially diluted two-fold, in V-well microplates. After incubation at 37°C for 60 mins, and centrifugation at 600 rpm for 3 mins, they were read macroscopically by tilting the plate at 70° and allowing negative reactions to trail. The degree of agglutination was graded in conventional manner and the titres given as the highest dilution giving complete agglutination (see Table IIIa). Anti-D titres were also determined by the IAG test using rabbit anti-human IgG and 3% R_1r , R_1R_1 , R_1^ur or R_O^ur cells in low ionic strength saline (LISS). The anti-D activity of the monoclonals against a panel of D variant red cells was also assessed using saline, albumin, papain and IAG (in LISS) tests. In a separate series of tests, the reactivity of the monoclonal anti-Ds of A1, A2, A3, B1, B3 and B11 with D^u red cells (15 sets of R_1^ur cells and 10 sets of R_2^ur cells, each set being taken from a different individual) was assessed by IAG (in LISS) using untreated supernatants. Using the same procedure, culture supernatant of B1 was further compared with culture supernatant of B2 against 1 set of R_1^ur cells and 3 sets of R_O^ur cells. The supernatants were tested on a Technicon Autogrouper 16C at dilutions ranging between 1:5 to 1:10,000.

Tests against cells of "normal" RhD positive or RhD negative phenotypes by albumin, papain and indirect antiglobulin techniques revealed that all the IgG1 monoclonal antibodies showed specificity for the D antigen (see Table VI below). None of

the monoclonal anti-Ds were reactive by saline. When tested by IAG against "partial" D positive cells, the antibodies agglutinated D^{IV}, D^V and D^{VII} red cells, but not D^{VI} or D^B (see Table VII below). As shown in Table VIII, the supernatants of A1, A2, A3, B1, B3 and B11 were all found to show stronger reactivity by IAG tests with 25 D^u cells (i.e. weak D) than polyclonal antiserum (the routine reagent employed by the South Western Regional Transfusion Service in the U.K). Using the same IAG test procedure, culture supernatant of B1 was found to exhibit similar or identical reactivity to culture supernatant of B2 against 4 further sets of D^u cells (see Table IX). All the IgG1 monoclonal antibodies reacted more strongly with R₁r (CDe/cde) or R₁R₁ (CDe/CDe) cells than with either D^u or D^V cells, though there were some differences in titres between the supernatants. When tested separately on a Technicon Autogrouper 16C, supernatants of A2, B1 and B11 could be used diluted to 1:1,000 for use as a routine anti-D and at 1:10 dilution to distinguish D^u from D negative cells.

Table VI

Serology of monoclonal anti-Rh(D) antibodies

RBC phenotype	R ₁ R ₁	R ₂ R ₂	R ₀ r	r'r'	r''r	rr
Saline 37°:		0	0	0	0	0
Albumin 37°C:		4-5	4-5	4-5	0	0
Papain 37°C:		5	5	5	0	0
IAG:		5	5	5	0	0

(Grade: 0 to 5)



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Table VII

Reaction of monoclonal anti-Rh(D) antibodies with "partial"
D positive RBCs by IAG

5	Monoclonal antibody:	A1	A2	A3	B1	B2	B3	B11
	D ^{IV} : (n=1)	5	5	5	5	4	3	5
	D ^V : (n=3)	5	5	5	5	5	5	5
10	D ^{VII} : (n=3)	5	5	5	5	5	5	5
	D ^{VI} : (n=3)	0	0	0	0	0	0	0
	D ^B : (n=5)	0	0	0	0	0	0	0

15 (Grade: 0 to 5)

Table VIII

Reactivity of monoclonal and polyclonal anti-Ds
with D^u RBCs by IAG

20 (15 R₁^ur and 10 R₂^ur)

25	Monoclonal antibody:	A1	A2	A3	B1	B3	B11	Poly-clonal anti-Rh(D) Serum
	Number with							
30	Grade 6:	16	15	11	6	1	5	0
	Grade 5:	8	9	12	16	7	17	4
	Grade 4:	1	1	2	3	9	3	12
	Grade 3:	0	0	0	0	4	0	7
	Grade 2:	0	0	0	0	4	0	1
35	Grade 1:	0	0	0	0	0	0	1

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Table IX

Monoclonal antibody	B1	B2
		(titres by IAG)
5 RBCs		
$R_1^u r$	512	512
$R_o^u r$	32	16
10 $R_o^u r$	512	512
$R_o^u r$	512	512

15

(iii) Lymphocyte ADCC assay

- Equal volumes (50 μ l) of target cells (chromium-51 labelled $R_1 R_1$ RBC suspension), effector cells (K lymphocytes) and anti-D culture supernatant were incubated overnight at 37°C in microplates after mild centrifugation, and the chromium-51 release measured (Urbaniak (1979) Br. J. Haematol. 42, 303-314). The effector:target cell ratio was 15:1.
- 25 The culture supernatants of clones B1, B2, B3, B11, B12 and B13 containing a monoclonal anti-Rh(D) of the Glm(3) allotype, unlike the other culture supernatants with antibody of the Glm(1,17) allotype, were found to be highly active in mediating
- 30 lysis of sensitised RBCs in the presence of K cells (see Table X below).

Table X

Monoclonal										Anti-Rh(D) serum
antibody:	A1	A2	A3	B1	B2	B3	B11	B12	B13	
<u>% specific</u>										
<u>lysis:</u>										
Effector KF	-2	-1	-2	91	*	99	83	*	*	95
Effector KL	*	13	*	*	78	*	*	*	*	*
Effector BW	5	4	3	56	*	73	70	*	*	92
Effector BK	4	15	*	91	*	*	81	71	75	*

* not determined

(iv) U937 Monocyte rosetting and phagocytosis assay

100µl packed OR₁R₂ RBCs were sensitised with 500µl anti-Rh(D) (previously adjusted to 1 µg/ml) at 37° for 60 minutes, washed and resuspended at 1 x 10⁸ cells/ml in RPMI. U937 cells were taken

5 in the log phase of growth and cultured for two days either in the presence or absence of interferon-γ (Amersham) at 50 U/ml. 45 x 10⁶ RBCs were then added to a pellet of 1.5 x 10⁶ U937 cells and mixed, giving a ratio of 30:1. For the rosetting assay,

10 the cells were incubated at room temperature for 5 minutes, spun at 600 rpm for 3 minutes and examined in a haemocytometer after a further 5-20 minutes. Phagocytosis was assessed immediately after incubating the cells at 37° for 3 hours. Results were expressed

15 as the percentage of monocytes with one or more adherent or phagocytosed RBCs.

A comparison of the results obtained with culture supernatants of clones A1, A2, A3, B1, B3 and B11 and a conventional polyclonal anti-Rh(D)

20 serum is given in Figures 1 and 2.

(v) Macrophage binding assay

RBCs (R₂r) (1 volume) were sensitised with monoclonal anti-Rh(D) (2 volumes of untreated culture supernatant) and incubated with monocyte-derived
 5 cultured macrophages. The macrophages were stimulated with 500 U/ml of recombinant immune interferon (Biogen, Geneva) during the 48 hours prior to their use in the assay. The binding of RBCs to macrophages was assessed microscopically and expressed as the
 10 macrophage binding index (= number of red cells attached to or ingested by 100 macrophages).

Table XI below shows the results obtained with culture supernatants of clones A1, A2, A3, B1, B3 and B11. The ability of these supernatants
 15 to bring about RBC-macrophage interaction was very poor compared to that of the polyclonal anti-Rh(D) serum (anti-Rh(D) - 43IU/ml), which served as a positive control.

Table XI

Source of anti-Rh(D)	A1	A2	A3	B1	B3	B11	polyclonal anti- Rh(D) serum.
Macrophage	20	28	24	5	20	17	416-
binding index							500

EXAMPLE 2Solution for Rh(D)-Phenotyping of RBCs

In general, it is preferred for the above purpose to use a blend of an anti-Rh(D) monoclonal antibody according to the invention (e.g. B11)

with a further IgG monoclonal antibody having anti- D^{VI} activity, for example the monoclonal antibody of the cell line B7 (ECACC 87091603) of our International patent application published under No. W089/02443 on 23

5 March 1989.

Solution for Manual Use

The final blend is 1:1:1 B11:B7: diluent.

10

Diluent 100 ml 30% Bovine Serum Albumin
2.42g KH₂PO₄
2.77g Na₂HPO₄·2H₂O
4.50g NaCl

15

0.2 ml Tween 20 (Trade Mark)

1.00g NaN₃

To 1.0 litre with distilled water: pH 6.8

20 This blend can be used in all manual tests for D and D^u typing, e.g. microtitre, microplate, IAG.

A blend of 1:1:2 B11:B7: diluent may also be used.

Blend for Machine Use

25

This blend may be used, for example, in a Technicon autogrouper 16C

Pre-blend reagents B11:B7 1:1

30

For D-Phenotyping (D-positive v. D-negative) the solution comprises 1:1000 blend: diluent, and for D^u determinations (D^u v. D-negative) the solution comprises 1:5 blend:diluent.

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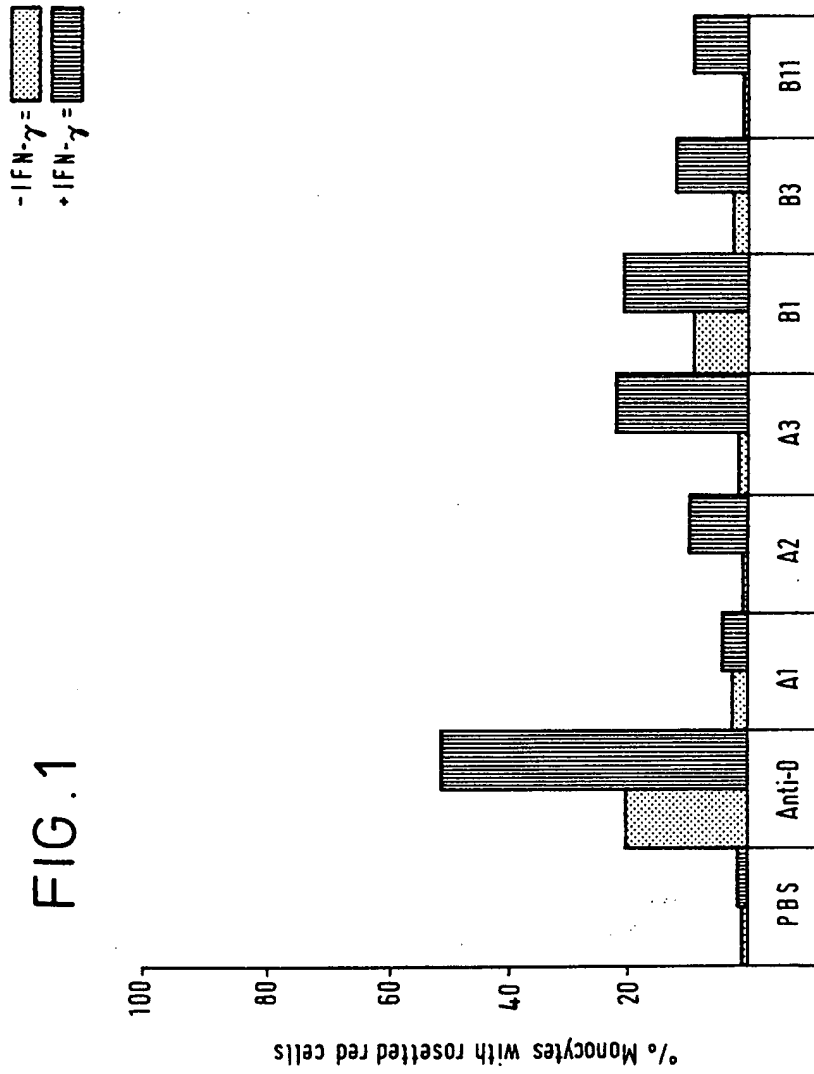
Diluent 2% Bovine Serum Albumin in 1.3% physiological saline containing 13.5% methylcellulose.

- 5 The monoclonal antibody B7 may be similarly blended with other antibodies of the present invention, e.g. the monoclonal antibodies of the deposited cell lines A3 (ECACC 87091605) and B2 (ECACC 87091604) to provide an anti-Rh(D) reagent with broad specificity for the
- 10 various D-variant antigens, including D^{VI}, and exhibiting activity against D^u cells by an IAG test.

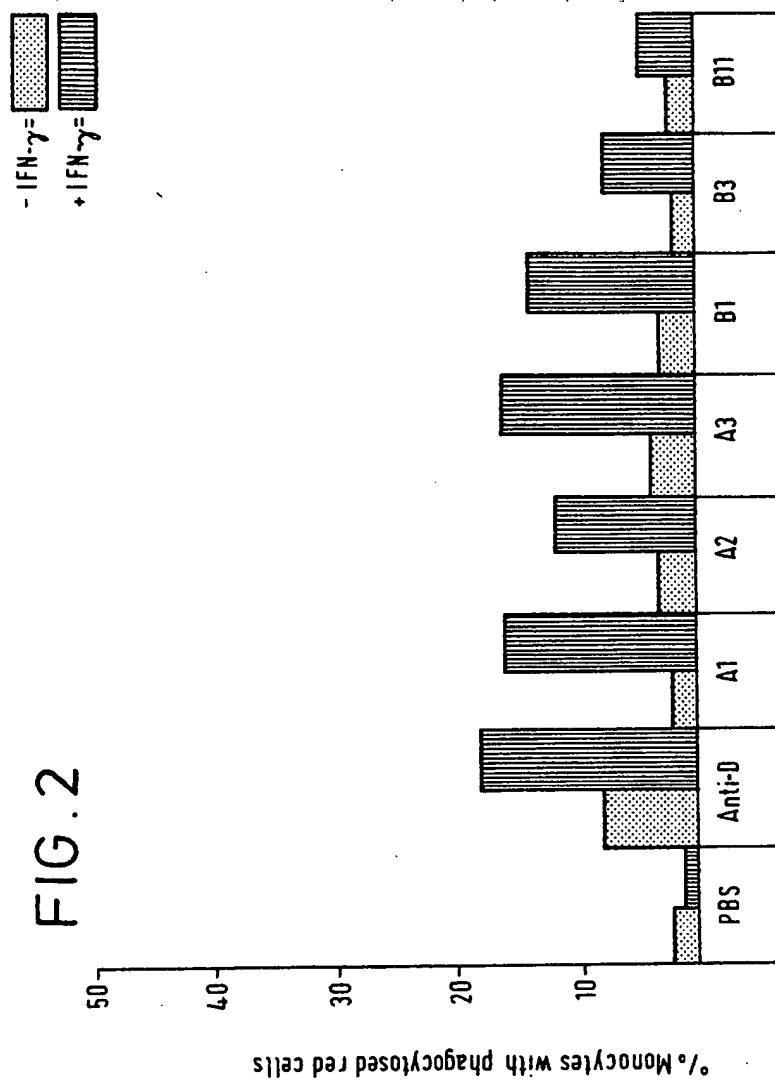


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